FLEXIBILITY OF BI- AND TRIANTENNARY GLYCANS OF THE *N*-ACETYLLACTOSAMINIC TYPE

A spin label study

Jean DAVOUST, Véronique MICHEL*, Geneviève SPIK*, Jean MONTREUIL* and Philippe F. DEVAUX

Equipe de Recherche Associée au CNRS no. 690, Institut de Biologie Physico-Chimique, 13, rue Pierre et Marie Curie, 75005 Paris and **Laboratoire de Chimie Biologique et Laboratoire Associé au CNRS no. 217, Université des Sciences et Techniques de Lille I, 59655 Villeneuve d'Ascq Cedex, France*

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1. Introduction

Glycans appear to be recognition signals in several systems where interactions between glycoproteins and cellular receptors are involved [1–3]. The study of the conformation and mobility of such glycans may provide information about their biological role. The construction of molecular models of human serotransferrin glycans suggests that the two trisaccharide sequences:
α-Neu Ac–(2 → 6)–β-Gal–(1 → 4)–β-GlcNac
present in external positions have a helical configuration and, like 'antennae' are mobile [4]. To confirm these dynamics properties, the application of physical methods constitutes an interesting approach. Various investigators have carried out ESR studies of spin-labeled glycans of immunoglobulins [5–7] and membrane glycoproteins [8–10]. Attention was drawn in those studies to the fast motion of the probes. To study the dynamics properties of oligosaccharides in erythrocyte membranes, optical probes have been used [11]: the fast motion was superimposed onto a slow motion which was attributed to cooperative movements of the oligosaccharide chains on the membrane surface [11].

Here, we describe the results obtained by spin-labeling sialic acid residues in two purified glycopeptides possessing bi- and triantennary glycans of known chemical structure. The ESR spectra have been recorded as a function of temperature in presence and absence of concanavalin A (con A). A fast motion of the probe is detected ($\tau < 10^{-9}$ s); however, analysis of the data obtained with doubly-labeled molecules, specifically labeled on sialic acid residues, allows us to demonstrate the existence of a relative motion between the chains associated with a large correlation time ($\tau_c \sim 10^{-6}$–10^{-7} s).

2. Materials and methods

2.1. *Preparation of the glycopeptides*

Sialic glycopeptides were prepared by extensive pronase digestions of human serotransferrin [2] and of Cohn fraction IV. The structure of the biantennary glycan of serotransferrin has been established [13]. In addition to the biantennary glycans, Cohn fraction IV contains a triantennary glycan, isolated and subjected to structural analysis by methylation, hydrazinolysis–nitrous deamination–mass spectrometry and ^{1}H NMR analysis at 360 MHz in [14].

2.2. *Labeling of the sialyl glycopeptides*

The method is based on the mild periodate oxidation of the *N*-acetylneuraminic acid residues leading to the formation of C_7 and C_8 derivatives, heptulosaminic and octulosaminic acids, respectively [15] and on the reactivity of the resulting aldehydic group with the amine extremity of 4-amino-2,2,6,6-tetramethylpiperidin-1-oxyl (Aldrich Chemical Co). The procedure used was as follows: glycopeptides were dissolved at 2 mM final conc. in 1 ml 0.1 M phosphate buffer (pH 7.4) containing 0.02 M sodium metaperiodate. After 10 min at 4°C in the dark, oxidation was stopped by the addition of an excess of ethylene glycol. The excess of periodate and the salts were removed by

passing the mixtures through a prepacked Pharmacia column (PD 10) equilibrated with distilled water. The eluted fractions were concentrated in a rotary evaporator at room temperature. Both spin-labeling and reduction of the resulting imine were done at room temperature for 24 h in 0.5 ml 0.1 M phosphate buffer (pH 7.4). The simultaneous addition of spin label and of solid sodium cyanoborohydride was performed by adding a 20-fold molar excess of each reagent with respect to the sialic content. The spin label was added from a 1 M stock solution in ethanol. Excess of reagents was removed by gel filtration on a Biogel P2 column. The spin-labeled glycopeptides were eluted with 0.1 M phosphate buffer (pH 7.4) and separated from free spin label as monitored by the ESR signal.

The glycopeptide concentration in each sample was estimated by titration of the neutral sugars using an orcinol sulfuric method [16].

The amount of C_7 and C_8 derivatives formed by the periodic oxidation of sialic acid, as well as the amount of non-oxidized *N*-acetylneuraminic acid, were determined by gas–liquid chromatography of sugars according to [17].

The spin label content of each glycan was calculated from the ratio of the amount of oxidized *N*-acetylneuraminic acid and of the amount of spin label calculated from its ESR spectrum at 20°C.

2.3. *Interaction with con A*

Concanavalin A (2.5 mg IBF, Clichy) was added to 20 nmol biantennary and 10 nmol triantennary glycopeptide samples dissolved in 1 ml 0.1 M phosphate buffer. After 2 min vortex mixing, the complexes were studied by ESR as described below. To check the specificity of the lectin–glycan interaction, the ESR spectra were compared to those obtained after dissociation of any con A–glycopeptide complexes by adding α-methyl-D-mannoside in a 10-fold molar excess with respect to the con A concentration.

2.4. *ESR and analysis of the spectra*

ESR experiments were performed with a Varian E109 spectrometer equipped with a field–frequency lock accessory and connected to a Tektronix 4051 computer used for data accumulation and spectral titration. The temperature of the sample was varied using a stream of nitrogen gas flowing through a dewar holding a 50 μl flat quartz cell. The temperature of the 50 μl sample volume was monitored continuously with a copper/constantan thermocouple. A radiofrequency power level of 10 mW and a detection modulation amplitude from 0.5–1 G were used. The spin label was systematically varied from 10–600 μM to avoid intermolecular spin–spin interactions.

Finally, the τ_c of the nitroxide residue was estimated whenever the spectrum consisted of narrow peaks using the formula derived in [18]. This leads to a crude estimate of a plausible anisotropic motion of the type experienced by a polysaccharidic chain.

3. Results

3.1. *ESR of the spin labeled glycopeptides*

The structures of the bi- and triantennary glycans and the amounts of oxidized and reduced *N*-acetylneuraminic acid residues are reported in table 1. After spin labeling, the elution of the covalently bound spin label from the Biogel P2 column was monitored by its ESR signal. Under these conditions, we have obtained a complete separation of the covalently bound spin label from the free spin label monitored by its characteristic ESR signal. The ESR spectra of the spin-labeled glycopeptides are given in fig.1. As a control experiment the same glycopeptides were treated in identical conditions with a non-reactive spin label, and no ESR signal was associated with the glycan elution peaks.

The amount of covalently bound spin label/glycopeptide is reported in table 1. These data indicate that under the previously described conditions, a quantitative and reproducible yield of labeling, close to a 1:1 ratio of bound spin label/sialic acid residue has been reached.

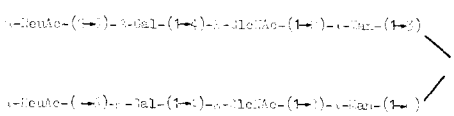
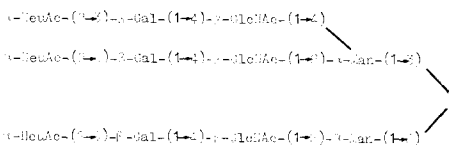
3.2. *Rotational mobility of the labeled glycopeptides*

Each of the two isolated spin-labeled glycopeptides used in this study gave rise to spectra, from 0–50°C, characteristic of a single fast rotating component as shown for the biantennary glycopeptide in fig.1 ($\tau_c = 0.63 \pm 0.09 \times 10^{-9}$ s at 20°C) whose overall line shape (peak ratio and line width) varied with temperature. This led to a temperature-dependent τ_c (fig.2). The Arrhenius plot of this rotational correlation shows an unique straight line for both glycopeptides from which a 6.9 ± 1.0 kcal/°C rotational activation energy was calculated.

3.3. *Intramolecular spin–spin interactions in bi- and triantennary glycans*

The spectra obtained at >20°C for both spin-labeled

Table 1
Spin label content of bi- and triantennary glycans

Structure of the glycans	A[a]	B[b]	C[c]	Ratio C/B	Ratio C/A
	650 ± 50	904 ± 30	690 ± 50	0.77 ± 0.11	1.3 ± 0.17
	307 ± 40	340 ± 30	290 ± 40	0.85 ± 0.15	2.2 ± 0.3

[a]Amount of each glycan (μmol); [b]amount of oxidized NeuAc in each glycan (μmol); [c]amount of spin label covalently bound to each glycan (μmol)

glycopeptides present not only a narrowing of the lines but also an enhancement in the ratio of the first two line peaks (fig.3 insert). This enhancement can be unambiguously attributed to spin–spin interactions that originate from a collision effect between probes [19]. Since these spectral abnormalities did not vanish after a 60-fold dilution of the spin-labeled glycopeptides, we attribute this effect to internal collisions, due to increased temperatures, between bi- and triantennary glycans, we systematically measured a spectral parameter (H_1/H_0) at all temperatures (fig.3). Spin-labeled triantennary glycan seems to undergo more pronounced intramolecular collisions. Its high value for the H_1/H_0 ratio indicates that intramolecular collisions originate from the supplementary branching of the triantennary glycan in contact with the two other branches, since both glycopeptides did bind an equivalent amount of spin label/sialic acid residue (table 1). Whenever the spin label is restricted to 1 sialic acid residue/glycopeptide (such as for a weak yield of labeling obtained in non-optimal reaction conditions), then the ratio H_1/H_0 is <1 at any temperature.

Over −2 to −10°C, the biantennary glycan spectra show fast rotating motion, although the aqueous buffer is frozen, as depicted in fig.4A for −10°C. The same

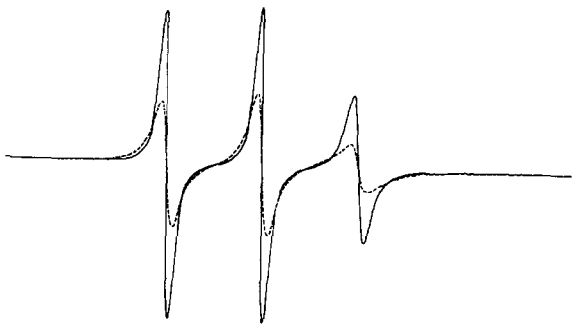


Fig.1. ESR spectra of spin-labeled biantennary glycopeptide at 20°C (——) in aqueous buffer (0.1 M sodium phosphate, pH 7.1); (– – –) after subsequent addition of con A. (Spectra are normalized to the same double integral.)

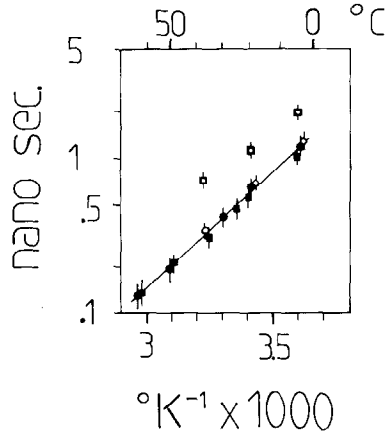


Fig.2. Arrhenius plot of the rotational correlation time estimated from ESR spectra of spin-labeled glycopeptides in aqueous buffer (0.1 M sodium phosphate, pH 7.1); triantennary glycopeptide; corresponding open symbols were obtained after addition of con A.

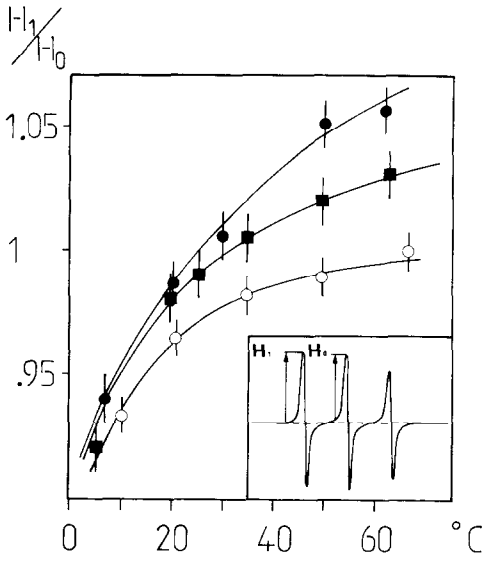


Fig.3. Temperature dependence of the index H_1/H_0 measured as depicted in the insert for spin-labeled glycopeptide. The error bars were deduced from an estimation of the signal to noise ratio: (■) spin-labeled biantennary glycopeptide; (●) spin-labeled triantennary glycopeptide; (○) bi- or triantennary glycopeptide with a spin label to sialic acid residue ratio <1.

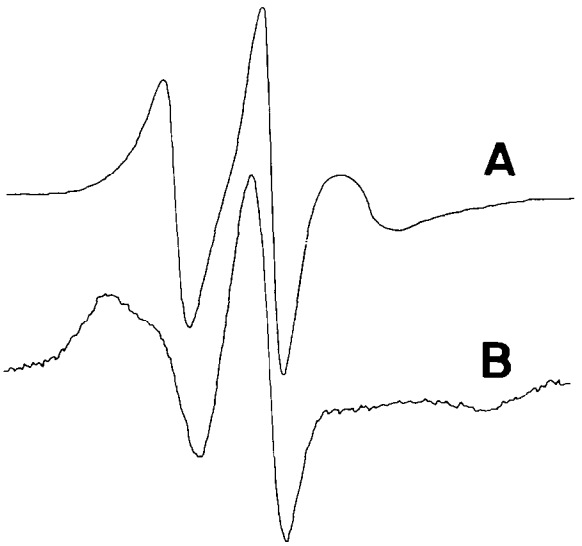


Fig.4. ESR spectra of spin-labeled biantennary glycopeptide in aqueous buffer (0.1 sodium phosphate, pH 7.1) below the freezing temperature: (A) −10°C; (B) −17°C.

glycan gives a spectrum characteristic of $\tau_c > 10^{-7}$ s at −17°C (fig.4B). This result way correspond to a defect of local freezing of water in the direct vicinity of the probe. We reached the same conclusion from the spectra obtained at <−2°C with the triantennary glycopeptide, except that it is already immobilized at −10°C, indicating again some dynamics difference between the two glycans.

3.4. *Modification of the motion due to interaction with con A*

The addition of con A in excess with respect to the mannose concentration in each sample slightly reduced the mobility of the probe bound to the biantennary glycan (fig.1); ($\tau_c = 1.10 \pm 0.15 \times 10^{-9}$ s at 20°C), but not that of the spin-labeled triantennary glycan. The addition of α-methyl-D-mannoside, which dissociates the complex, reversed the τ_c to its original value found without lectin. This result suggests that the specific interaction of con A binding sites with biantennary glycans [20,21] slightly increases its overall τ_c. However, the value measured for the rotational correlation time (fig.2) cannot be representative of the overall rotational motion of the biantennary glycan–lectin complex, since the M_r of tetrameric con A could only give rise to a much smaller characteristic rotational time.

4. Discussion

Here, a spin label is covalently attached to a sialic residue at the extremity of oligosaccharide chains of bi- and triantennary glycopeptides. The ESR spectra indicate a high mobility of the probe ($\tau < 10^{-9}$ s), confirming the work in [9] using an identical spin-labeling procedure on glycophorin, and that in [11] measuring the mobility of a fluorescent probe attached to oligosaccharide chains of human erythrocytes.

In all of these experiments part of the fast motion can be attributed to free rotation of the probe itself. Therefore, such spin-label (or fluorescent) studies report primarily on the viscosity of the immediate environment of the probe (i.e., on the aqueous phase). Here, as in [9], the order parameter of fast motion is practically 0. However, in [11] the order parameter was close to 0.8 [11]. This difference can be explained by the large size of the eosin probe used in [11]. In spite of the large degree of freedom of our probe, we now show that it is capable of reporting on the inter-

nal rigidity of the oligosaccharide molecule, as well as on 'slow motion' taking place within the glycopeptide.

4.1. *The spectral modifications induced by con A binding indicates a certain rigidity of the oligosaccharide chains*

In the presence of con A the spectrum of the spin-labeled biantennary glycan is modified; this shows that the interaction of the lectin with the glycan is not inhibited by the presence of the nitroxide. We want to emphasise that the spectrum obtained does not correspond to a mixture of slow and fast motion, which would result from a partial binding with a low affinity. On the contrary, the spectrum (fig.1,———) is homogeneous. It corresponds to an increase of correlation time by a factor of ~2; the order parameter shows that the nitroxide is not entrapped in the con A molecule. This conclusion may have been anticipated since con A is thought to bind to the β-Glc-NAc–(1 → 2)–Man residue [21]. Nevertheless, the immobilization of this residue is propagated at the level of the terminal residue, since the spectrum of the spin-labeled sialic acid is modified. This is only possible providing a certain rigidity of the oligosaccharide chain exists.

4.2. *Spin–spin interaction at high temperature indicates a slow motion between oligosaccharide chains*

If, on the average, >1 chain of bi- and triantennary glycans are labeled, spin–spin interactions can be detected at >35°C (fig.3). This spin–spin interaction is not due to intermolecular interactions since dilution does not affect the phenomenon. Furthermore, since the labeling is very specific in our experiment, only one spin label can be fixed per chain. Thus evidence of spin–spin interaction indicates that an interaction takes place between 2 chains of the same molecule. Because the interaction takes place at high temperature, and is not visible at low temperature, it is essentially due to Heisenberg spin exchange and not to static dipole–dipole interaction [19]. The interaction is therefore due to increasing relative motion between 2 adjacent chains. The time scale of this motion is relatively well defined. Heisenberg spin exchange is detectable with nitroxides when the exchange rate is ⩾1 MHz. The type of spectra observed in our experiments at 60°C corresponds to an exchange rate of a few MHz. Thus an intramolecular motion with $\tau_c = 10^{-6}$–10^{-7} s is shown in this experiment. This motion is much too slow to correspond to the rotation of the piperidine rings. We attribute this motion to a change in the conformation of the bi- and triantennary glycans which can be considered as an overall flexibility [4].

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